ORIGINAL ARTICLE

Comparative study of periodontal differentiation propensity of induced pluripotent stem cells from different tissue origins

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Abstract

Background: Despite being almost identical to embryonic stem cells, induced pluripotent stem cells (iPSCs) have been shown to possess a residual somatic memory that favors their differentiation propensity into donor tissue. To further confirm this assumption, we compare for the first time the periodontal differentiation tendency of human gingival fibroblast-derived iPSCs (G-iPSCs) and human neonatal skin fibroblast-derived iPSCs (S-iPSCs) to assess whether G-iPSCs could be more efficiently induced toward periodontal cells.

Methods: We induced G- and S-iPSCs under the treatment of growth/differentiation factor-5 and connective tissue growth factor, respectively, for 14 days. Immunofluorescence staining and real-time polymerase chain reaction were used to compare their expression levels of related markers. Furthermore, a hydrogel carrier was developed to seed these periodontal progenitors for subcutaneous implantation in non-obese diabetic-severe combined immunodeficiency disease mice. Their differentiated periodontal phenotype maintenance was further assayed by HE observation, immunohistochemical staining and immunofluorescence co-localization with pre-labeled PKH67.

Results: As expected, both iPSCs were inclined to differentiate back into their original lineage by expressing higher markers at both gene and protein levels in vitro. HE observation of G-iPSCs-seeded hydrogel constructs present more mineralized structure formation than S-iPSCs-seeded ones. Immunohistochemical staining and immunofluorescence analysis also showed stronger positive staining for periodontal related markers in G-iPSCs-seeded hydrogel constructs.

Conclusions: Our results preliminarily confirmed that both G- and S-iPSCs were inclined to differentiate back into their original tissue in vitro. Animal study further confirmed the phenotype maintenance of periodontal differentiated G-iPSCs, which highlighted their significant implications for therapeutic use in periodontal regeneration.

KEYWORDS
growth factors, induced pluripotent stem cells, tissue engineering

To date, one of the most prominent trends[1] in periodontal tissue regeneration has been the application of induced pluripotent stem cells (iPSCs). The discovery of iPSCs—somatic cells that are reprogrammed back to pluripotency via the introduction of defined transcription factors[2]—has provided new insights into regenerative therapy. Although almost identical to embryonic stem cells (ESCs) in self-renewal capacity and differentiation plasticity,[3] evidence for a retained donor phenotype memory by iPSCs has been demonstrated in several studies on global gene expression and histone modification.[4] Kim et al. reported that incomplete promoter DNA methylation may partially explain the residual expression pattern of tissue-specific genes and related this epigenetic memory of murine and human iPSCs to their differentiation behavior.[5,6] Other reports also demonstrated that iPSCs are more inclined to differentiate back into their original lineage.[7–11] This inherent characteristic of iPSCs has since been exploited: iPSCs originating from the exact tissue to which they will be applied downstream are used in order to achieve rapid and efficient differentiation.[8,12,13]

There have been some contradictory findings surrounding the inclined differentiation propensity back into their original cell type via epigenetic memory. Variations in directed differentiation potential may also be attributed to genetic variability,[14–17] clonal differences,[18] and iPSC quality.[19] While differentiation biases among iPSCs may be exploited for deriving certain tissues, they may not provide a universal differentiation advantage for all somatic cells. It has been shown that the differentiation potential of skeletal muscle- and hepatic lineage-derived iPSCs is not skewed[20,21] and thus the question whether iPSCs retain an epigenetic memory and whether they tend to redifferentiate back to their former identity is still a matter of debate which requires further investigation.

In the meantime, although great progress has been made in iPSC-based tissue engineering (e.g., cardiomyocyte- and hepatocyte-specific differentiation),[22] only few cases of the application of iPSCs in periodontal regeneration have been reported[23] and the functional incorporation of PDL, alveolar bone, and cementum tissue remains a challenge. Ideal regenerated PDL fibers should insert into newly formed cementum and alveolar bone to enable a degree of movement for periodontal homeostasis and repair.

Growth /differentiation factor-5 (GDF-5)[24] is a subfamily of TGF-β superfamily, also known as cartilage morphogenetic protein, which regulates cell differentiation and promotes formation of bone, cartilage, and ligament. It is also expressed in the process of root development and periodontal ligament formation.[24] In addition, GDF-5 can significantly promote the proliferation of periodontal ligament cells and the synthesis of glycosaminoglycan in a dose-dependent mode.[25] Histological and clinical studies have shown that GDF-5 can promote the healing and regeneration of periodontal defect[26,27] Our previous study demonstrated that GDF-5 could induce iPSCs to differentiate into osteoblasts, periodontal ligament cells and cementum tissue both in vitro and in vivo, and explored the optimal induction concentration was 200 μg/L.[28]

Connective tissue growth factor (CTGF) is a kind of cysteine-rich secretory protein polypeptide, found in human umbilical vein endothelial cell conditioned medium.[29] Besides promoting osteogenesis and chondrogenesis,[30] this factor can achieve fibrosis by promoting cell proliferation and transformation, regulating extracellular matrix expression, and mediating cell adhesion and migration.[31] A number of studies[32–34] have shown that CTGF can induce bone marrow mesenchymal stem cells to differentiate into skin fibroblasts, by expressing related markers and synthesizing various collagen. It can promote the fibrosis of connective tissue in a rat model with an optimal induction concentration of 100 ng/mL.[32,33]

Scaffold material is a three-dimensional porous structure with good biocompatibility and biodegradability, which can promote cell adhesion, proliferation, differentiation, and metabolism. Commonly used scaffold materials include natural polymers, synthetic polymer materials,[35] and crystalline ceramic materials, etc.[36] Hyaluronic acid (HA) is an important component of natural extracellular matrix that maintains the viscoelasticity of connective tissue, while promoting wound healing and cell migration, proliferation, and differentiation.[37] HA hydrogels can achieve in situ filling by injection and create an ideal microenvironment for the growth and differentiation of seed cells. It has been widely used as the scaffold material for bone, cartilage, heart, and nerve tissue regeneration, and has also been shown to possess good biocompatibility and biodegradability in periodontal regeneration.[38–40]

The aim of this study was to assess, for the first time, whether gingival fibroblast-derived iPSCs (G-iPSCs) could be more efficiently induced to differentiate into functional periodontal cells through an embryonic body (EB)-mediated routine upon GDF-5 exposure, hence providing more evidence for the beneficial exploitation of epigenetic memory in regenerative therapy. While most comparative studies focus on a one-way authentication for a certain differentiation direction, we directed both G- and S-iPSCs into skin tissue to see whether S-iPSCs showed a similar differentiation tendency back to their cell of origin to confirm our assumption of somatic memory. A hydrogel carrier was furthermore developed to seed G-iPSC-derived periodontal progenitors, and the resulting differentiated phenotype maintenance was assayed in a subcutaneous animal model in vivo.

TABLE 1 List of primer sequences used for real-time PCR

Primer	Forward	Reverse
GAPDH	5′-AGGTCGGTGTGAACGGATTTG-3′	5′-TGTAGACCATGTAGTTGAGGTCA-3′
BSP	5′-CAGGGAGGCAGTGACTCTTC-3′	5′-AGTGTGGAAAGTGTGGCGTT-3′
Periostin	5′-TGGAGAAAGGGAGTAAGCAAGG-3′	5′-TTCAAGTAGGCTGAGGAAGGTG-3′
CEMP1	5′-GGGCACATCAAGCACTGACAG-3′	5′-CCCTTAGGAAGTGGCTGTCCAG-3′
COL1A1	5′-GGACACAATGGATTGCAAGG-3′	5′-TAACCACTGCTCCACTCTGG-3′
COL3A1	5′-TTGGGTTGTCTAATATGGT-3′	5′-TCTCAGGATTTGTAGGGAT-3′

1 | MATERIALS AND METHODS

1.1 | Cell preparation and two-way specific differentiation in vitro

Human iPSCs derived from gingival tissues were obtained as previously described.[28] The gingival tissues were harvested from the extracted teeth of systemically and periodontally healthy donors (18 to 30 years old) who sought for orthodontic therapy or third molar removal surgery at Peking University Hospital of Stomatology. Donors all signed informed consents. The protocols were approved by the Biomedical Ethics Committee, Peking University Hospital of Stomatology (Approval No. PKUSSIRB-201414048). Human neonatal skin fibroblast-derived iPSCs (CA4002106 hiPSC) were purchased from the manufacturer. Both iPSCs were generated in identical ways and pluripotency was validated for each cell type, specifically by assessing colony morphology, growth rate, marker expression, EB formation, and teratoma development, as is shown in our previous study and on the official manufacturer's website. All iPSCs were cultured on basement membrane matrix gel*-coated 60-mm dishes and were passaged to the same generation in hES/iPS maintenance culture medium.[†]

To examine the differentiation propensity of iPSCs, EB formation was induced and assessed. In brief, iPSCs were suspended in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12)[‡] containing 20% FBS.[§] 0.1 mM β-mercaptoethanol,[¶] 1 mM L-glutamine,[¶] and 1% non-essential amino acid[§] on non-coated dishes. The cells were cultured in suspension for 5 days.

The EBs grown from the iPSCs were transferred to 0.01% matrix gel-coated dishes and cultured in DMEM/F12 basal medium containing 20% FBS and 200 ng/mL recombinant human growth/differentiation factor-5 (GDF-5)[#] to

induce periodontal differentiation according to our published protocol.[28] For skin directed differentiation, EBs were grown on matrix gel-coated dishes in DMEM/F12 basal medium containing 20% FBS and 100 ng/mL connective tissue growth factor (CTGF).[∥][32,33] EBs cultured in DMEM/F12 containing 20% FBS for spontaneous differentiation were used as self-controls. Culture media were replenished every other day, and images were captured under an inverted microscope.

1.2 | Real-time polymerase chain reaction

After 14 days of incubation, real-time polymerase chain reaction(PCR) was used to assess the expression of genes representative of bone (bone sialoprotein [BSP]), PDL connective tissues (periostin), and cementum (cementum protein 1 [CEMP1])[41–43] as an indicator of periodontal differentiated cells. Expression of skin-related genes (Collagen I [COL1A1] and Collagen III [COL3A1])[44] was evaluated in skin induction assays. Total RNA was extracted using an RNAeasy kit,[**] and cDNA was synthesized using a cDNA synthesis kit.[††] Real-time PCR was performed in a qPCR System[‡‡] using a quantitative PCR reagent kit.[††] Relative quantification of gene expression was carried out using the comparative cycle threshold (Ct) method with GAPDH as an internal control. The primer sequences are listed in Table 1.

1.3 | Immunofluorescence staining

After 14 days of induction, all cells were fixed in 4% paraformaldehyde[§§] for 30 minutes, treated with 0.5% Triton X-100 for 45 minutes, then washed with PBS three times. Cells were then immersed in 3% bovine serum albumin (BSA)[¶¶] for 1 hour at room temperature. After removal of the blocking buffer, iPSCs were incubated overnight at room temperature with primary antibodies[##] against BSP, periostin,

or CEMP1[##] at a dilution of 1:50, 1:200, and 1:100, respectively, to assess periodontal differentiation by the iPSCs. Skin differentiated iPSCs were incubated with primary antibodies[##] against Collegen-1 (COL-1) and Collegen-3 (COL-3) at a dilution of 1:100. All samples were then incubated with secondary antibodies conjugated with phalloidin* or wheat germ agglutinin* at a dilution of 1:200 for 1 hour. Spontaneous differentiation groups were used as negative controls. Nuclei were stained using mounting medium with DAPI[†] and images were captured by confocal laser scanning microscope (CLSM).[‡]

1.4 | Subcutaneous implantation of hydrogel-encapsulated iPSCs in an animal model

Commercially available HyStem-C hydrogels[§] were prepared according to the manufacturer's instructions. Briefly, degassed, deionized water was used to dissolve Glycosil (thiol-modified hyaluronan), Gelin-S (thiol-modified collagen), and Extralink (thiol-reactive PEGDA crosslinker) in individual vials. The periodontal progenitors derived from G-iPSCs and S-iPSCs were purified and pre-labeled with PKH67 Green Fluorescent Cell Linker.[¶] Subsequently, equal volumes of Glucosil and Gelin-S were mixed prior to the seeding of pre-labeled cells (5×10^6 cells/mL). To form the hydrogel, Extralink was added to the mixture in a 1:4 ratio.

Three intervention groups were included: 1) periodontal progenitors from G-iPSCs + hydrogel, 2) periodontal progenitors from S-iPSCs + hydrogel, and 3) hydrogel only. The hydrogel constructs (n = 3 for each group) were implanted into subcutaneous pockets on the dorsal surface of 6- to 8-week-old male non-obese diabetic-severe combined immunodeficiency disease mice[¶] under abdominal anesthesia with sodium pentobarbital[¶] Animal ethics approval was granted by the Biomedical Ethics Committee of Peking University (Approval No. LA 2014163). After 6 weeks, all mice were sacrificed, and the implants were harvested for further analysis.

1.5 | Histological analysis of harvested specimens

The harvested specimens were fixed in 4% paraformaldehyde overnight, embedded in paraffin, and processed for hematoxylin/eosin (HE) staining. For immunohistochemical staining, a cell and tissue staining kit (HRP-AEC system)[ǁ]

was used according to the manufacturer's instructions. Sections were deparaffinized, rehydrated, and then immersed in 3% H_2O_2 for 10 minutes to quench the endogenous peroxidase activity. After blocking, sections were incubated with primary antibodies against BSP, periostin, and CEMP1 with a dilution of 1:400, 1:500, and 1:200, respectively. Staining was visualized by the HRP-AEC reaction after further incubation with secondary antibodies at a dilution of 1:200 and images were captured under an inverted microscope.[#]

For immunofluorescence staining, the harvested specimens were fixed with 4% paraformaldehyde for 15 minutes and sectioned at a thickness of 5 mm. After serum blocking, sections were stained with primary antibodies against BSP, periostin, and CEMP1[##] at a dilution of 1:50, 1:200, and 1:100, respectively, and then incubated with Alexa Fluor 594-conjugated secondary antibody* at a dilution of 1:200. Nuclei were counterstained using DAPI Fluoromount.[†] Images were captured under CLSM.[‡] All differentiated iPSCs were PKH67[¶]-labeled before implantation to assess the survival and localization of transplanted cells. The percentage of positive stained cells in fluorescence-labeled donor cell populations was calculated for all implants (n = 3).

1.6 | Statistical analysis

At least three samples were used for each quantitative experiment. Mean values were entered for statistical analysis with IBM SPSS Statistics 20.0, and data were expressed as mean ± SD. Differences between groups were assessed by Student's t-test. Significance was set at $P < 0.05$ with n indicating the number of independent experiments.

2 | RESULTS

2.1 | Characteristics of iPSCs and two-way specific differentiation in vitro

The in vitro experimental design is illustrated in Figure 1A. Both iPSC clones maintained morphological and proliferation characteristics of ESCs after passaging. After 5 days of floating cultivation, spherical EBs were formed in vitro. After 14 days of two-way specific induction, morphologically homogeneous populations dominated both cell cultures, showing a very similar morphology to spindle-shaped fibroblasts (Figure 1B).

2.2 | Real-time polymerase chain reaction

To investigate the differentiation capacities of G-iPSCs and S-iPSCs, real-time PCR was used to detect periodontal
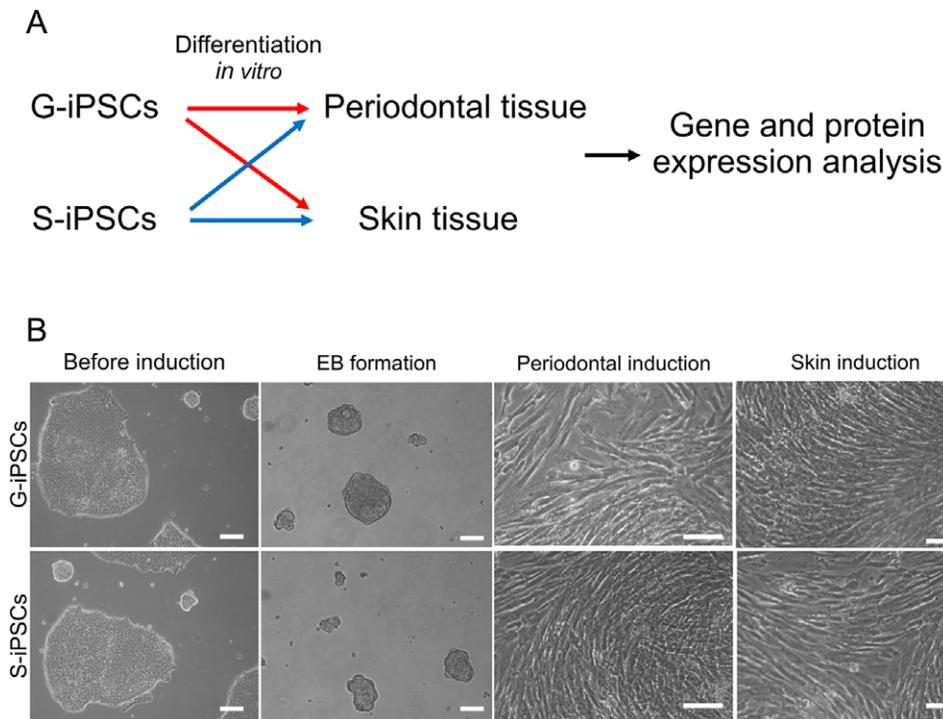
FIGURE 1 Specific two-way differentiation potential of iPSCs in vitro. (A) Experimental design. Gingival fibroblast-derived iPSCs (G-iPSCs) and skin fibroblast-derived iPSCs (S-iPSCs) were respectively differentiated toward periodontal and skin tissue in vitro. (B) Representative light microscopy images of the two iPSCs before induction and after EBs were formed for further differentiation. Scale bar = 100 μm

markers (*BSP, periostin, CEMP1*) and skin markers (*COL1A1* and *COL3A1*) after 14 days of specific differentiation. G-iPSCs were shown to express significantly higher levels of *BSP, periostin,* and *CEMP1* than S-iPSCs upon exposure to GDF5 (*BSP*: $t = 2.125$, $P < 0.05$; *periostin*: $t = 2.246$, $P < 0.05$; *CEMP1*: $t = 3.754$, $P < 0.05$); however, no significant difference in *periostin* expression was detected among the two iPSC cell lines in spontaneous differentiation controls ($t = 1.876$, $P > 0.05$) (Figure 2A). After 14 days of CTGF induction, mRNA expression of *COL3A1* was significantly enhanced in S-iPSCs compared to G-iPSCs ($t = 3.933$, $P < 0.05$), indicating that S-iPSCs were more inclined to differentiate toward the skin lineage. The *COL1A1* expression in both the induction and control groups, however, did not differ ($t = 1.697$, $P > 0.05$) (Figure 2B).

2.3 | Immunofluorescence staining

Fluorescence intensity analyzed by Image J software revealed stronger staining of BSP and CEMP1 in cultures originating from G-iPSCs compared to those originating from S-iPSCs in periodontal assays (BSP: $t = 2.584$, $P < 0.05$; CEMP1: $t = 3.493$, $P < 0.05$). In accordance with gene expression data, S-iPSCs were comparable to G-iPSCs in terms of periostin expression ($t = 1.245$, $P > 0.05$). Strong Collegen-1 and Collegen-3 staining was observed in S-iPSCs upon exposure to CTGF, whereas G-iPSCs displayed markedly weaker staining of both markers (Collegen-1: $t = 4.084$, $P < 0.05$;

Collegen-3: $t = 3.257$, $P < 0.05$). As expected, staining was negative in all negative controls (Figure 3).

2.4 | Subcutaneous implantation of hydrogel-encapsulated iPSCs in an animal model

To examine whether the G-iPSC-derived periodontal progenitors were functional, animal studies were performed as a means of evaluating phenotype maintenance. Three intervention groups were included: 1) periodontal progenitors from G-iPSCs + hydrogel, 2) periodontal progenitors from S-iPSCs + hydrogel, and 3) hydrogel only. After composites were transplanted subcutaneously in NOD/SCID mice for 6 weeks, all implants were retrieved. No adverse local responses (e.g., inflammation, teratoma formation) were observed.

2.5 | Histological analysis of harvested specimens

2.5.1 | H&E staining

The histology of a representative implant showed uniform cell growth inside the scaffold and bright pink staining regions indicating mineralized tissue. Compared with the S-iPSCs, the G-iPSCs showed a higher tissue regenerative capacity indicated by the production of more mineralized structures. In contrast, S-iPSCs transplants had mainly formed connective tissue with relatively small amounts of mineralized tissue
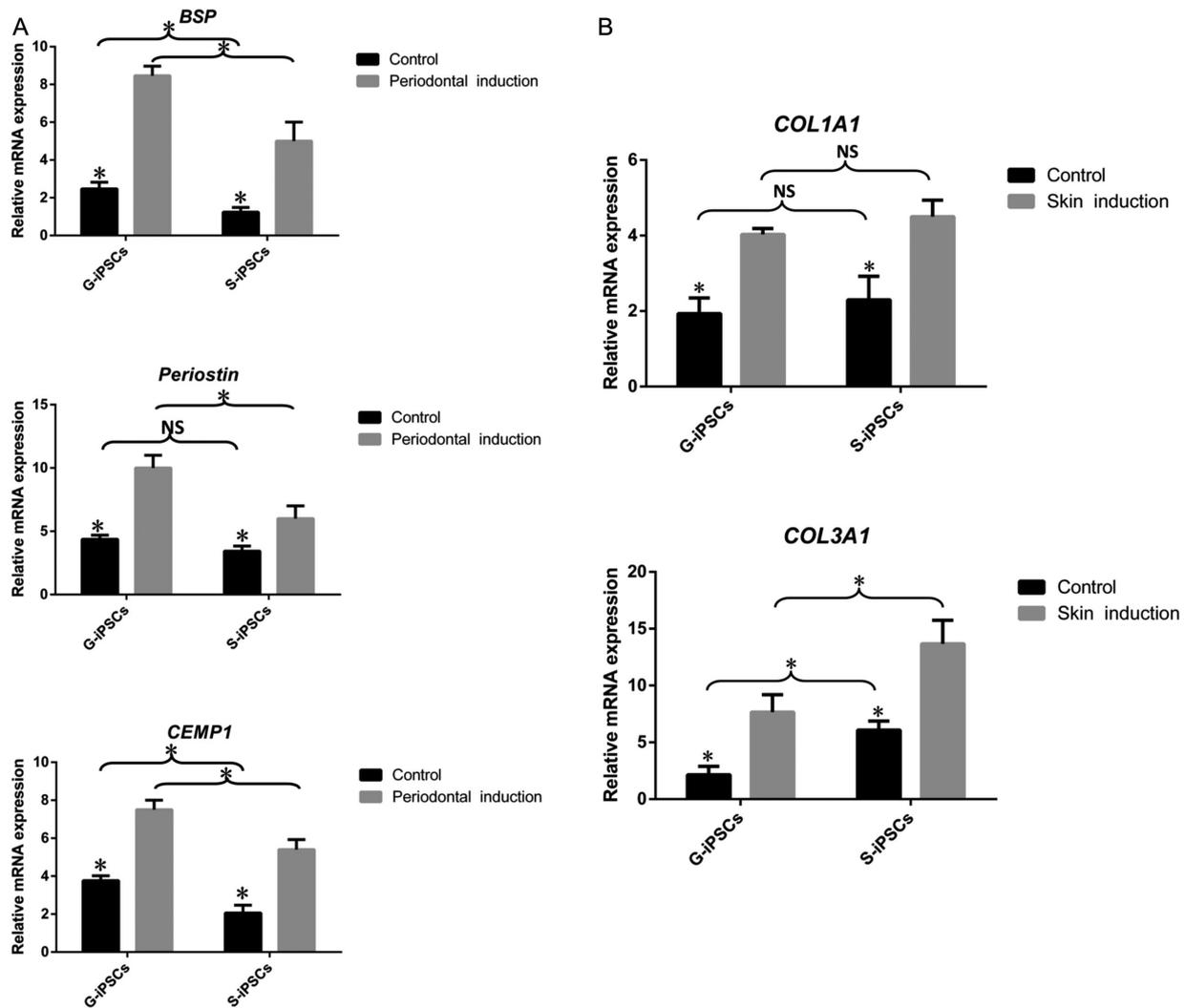
FIGURE 2 Real-time polymerase chain reaction (PCR) analysis (A) G-iPSCs expressed significantly higher levels of BSP, periostin, and CEMP1 than S-iPSCs upon exposure to GDF5 (BSP: t = 2.125, $P < 0.05$; periostin: t = 2.246, $P < 0.05$; CEMP1: t = 3.754, $P < 0.05$); however, no significant difference in periostin expression was detected in spontaneous differentiation controls (t = 1.876, $P > 0.05$). B) Expression of COL3A1 was significantly enhanced in S-iPSCs (t = 3.933, $P < 0.05$), while the COL1A1 expression in both groups did not differ (t = 1.697, $P > 0.05$). The error bars denote the mean $\pm$ SD of three individual experiments. *$P < 0.05$ (unpaired t-test)

lining the hydrogel surfaces. Cells embedded within the mineralized structures appeared to be osteocyte-like cells morphologically, indicating that G-iPSC-derived cells had contributed to the formation of new bone. No teratoma tissue formation was observed (Figure 4A).

2.5.2 | Immunohistochemistry staining

Immunohistochemical staining of G-iPSCs-seeded constructs revealed stronger positive staining for anti-BSP and anti-CEMP1 antibodies than S-iPSCs transplants analyzed by Image-Pro Plus (IPP) software. (BSP: $t = 4.707$, $P < 0.05$; CEMP1: $t = 6.708$, $P < 0.05$) However, their OD values for periostin expression did not differ ($t = 0.7276$, $P > 0.05$). In contrast, hardly any positive staining was detected in the hydrogel only implants because of a lack of proper

differentiated cells in the subcutaneous healing microenvironment (Figure 4B).

2.5.3 | Immunofluorescence staining

To track donor cells of specimens, differentiated progenitors labeled with PKH67 fluorescent cell linker kits were also examined by immunofluorescence staining. Observations for the colocalization of markers of interest with PKH67 correlated well with the immunohistochemical findings: G-iPSC-derived periodontal cell populations are better than their S-iPSC-derived counterparts at maintaining periodontal phenotype in an animal model with a significantly stronger fluorescence intensity analyzed by Image J software (BSP: $t = 8.552$, $P < 0.05$; periostin: $t = 3.280$, $P < 0.05$; CEMP1: $t = 4.902$, $P < 0.05$). Their proportions
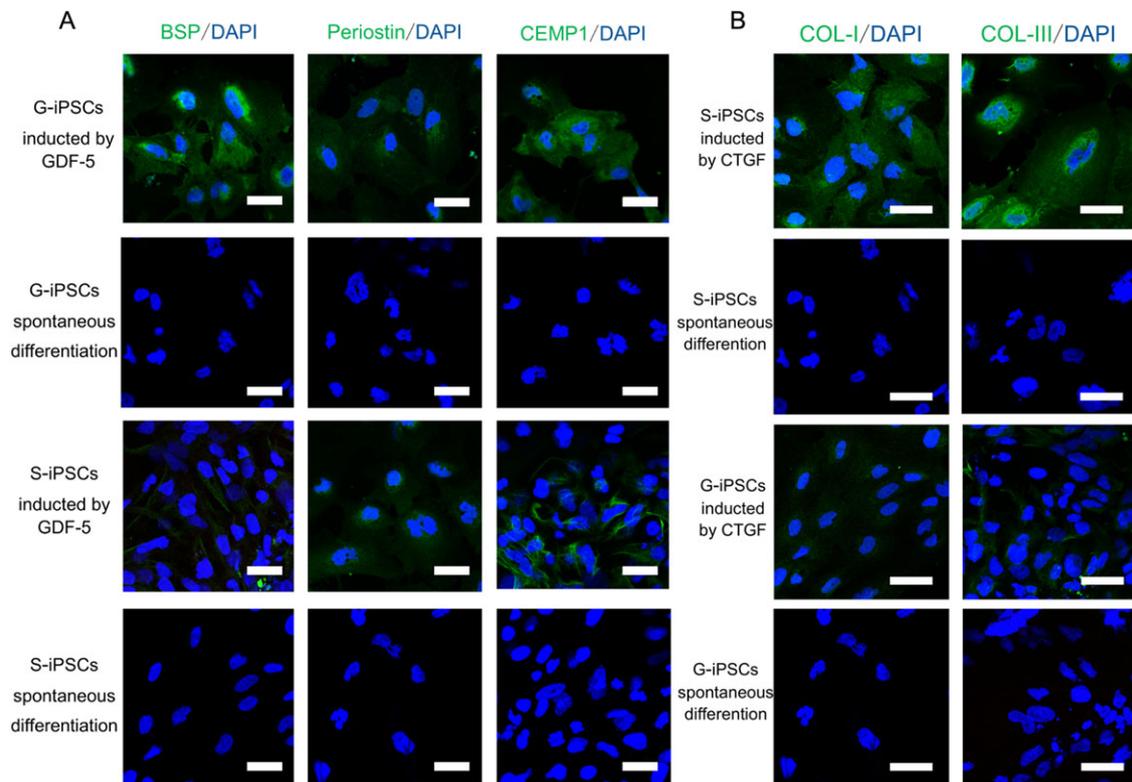
FIGURE 3 Representative sections depicting immunofluorescence staining. A) Fluorescence intensity analyzed by Image J software revealed stronger staining of BSP and CEMP1 in cultures originating from G-iPSCs compared to those from S-iPSCs in periodontal assays (BSP: t = 2.584, $P < 0.05$; CEMP1: t = 3.493, $P < 0.05$), while both iPSCs were comparable in terms of periostin expression (t = 1.245, $P > 0.05$). B) Strong COL-1 and COL-3 staining was observed in S-iPSCs upon exposure to CTGF, whereas G-iPSCs displayed markedly weaker staining (COL-1: t = 4.084, $P < 0.05$; COL-3: t = 3.257, $P < 0.05$). Staining was negative in all negative controls. Scale bar = 50 μm
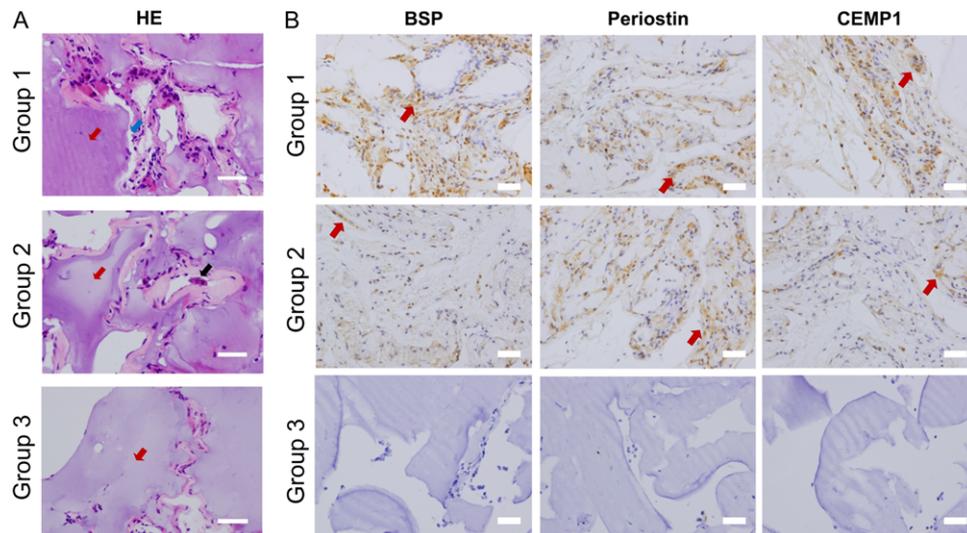

FIGURE 4 Harvested specimens 6 weeks after subcutaneous transplantation in NOD/SCID mice. A) HE staining of G-iPSCs-seeded constructs formed more mineralized structures (red arrow) than S-iPSCs-seeded ones, and cells embedded within the mineralized structures appeared to be osteocyte-like cells (blue arrow). Bone lacuna could be seen in S-iPSCs-seeded constructs (black arrow). B) Immunohistochemical staining of G-iPSCs-seeded constructs revealed stronger positive staining (red arrow) for anti-BSP and anti-CEMP1 antibodies analyzed by Image-Pro Plus (IPP) software. (BSP: t = 4.707, $P < 0.05$; CEMP1: t = 6.708, $P < 0.05$) Their OD values for periostin expression did not differ (t = 0.7276, $P > 0.05$). The unseeded scaffold control did not yield any positive staining. Scale bar = 100 μm. (Group 1: periodontal progenitors from G-iPSCs + hydrogel; Group 2: periodontal progenitors from S-iPSCs + hydrogel; Group 3: hydrogel only.)

of PKH67-labeled donor cells expressing BSP, periostin, and CEMP1 were 92.36% ± 8.52%, 78.21% ± 9.86%, and 86.58% ± 11.33%, respectively (n = 3 technical replicates). By contrast, expression of these markers was not detected in the ingrown indigenous cells in hydrogels (Figure 5). These data collectively demonstrate the high efficiency of periodontal regeneration achieved with G-iPSCs-seeded hydrogel composites subcutaneously engrafted in an animal model.

3 | DISCUSSION

Post-natal somatic cells are characterized by low proliferation rates, which limit their use for replacement therapy. Accordingly, adult stem cells (ASCs) such as human gingival mesenchymal stromal cells (GMSCs) and periodontal ligament stem cells (PDLSCs) have been suggested as new candidates for periodontal regeneration as they have greater survival rates and undergo cell replication.[45,46] Whether or not functional alveolar bone with periodontal ligament and cementum can be obtained from ASCs remains controversial, for there has been no existing study showing morphological evaluation of ASCs converted to functional periodontal structures. Embryonic stem cells (ESCs)[1] constitute a more reliable source of functional periodontal cells, as they are capable of maturing into EBs that can generate all three germ layers when cultured in vitro. The use of human ESCs, however, is hampered by their immunogenicity (which happens to be lower than that of ACSs) and more importantly by ethical restrictions regarding their derivation from blastocysts or early epiblasts.

Apart from certain drawbacks such as carcinogenicity and low reprogramming efficiency, iPSCs offer a number of advantages over ESCs: immune rejection and ethical concerns are avoided,[47] and iPSCs yield homogeneous cellular populations with prolonged self-renewal and higher plasticity. iPSCs have, therefore, become a novel ideal cell source for periodontal regenerative medicine. Although almost identical to ESCs in proliferation and differentiation capacity, different tissue-derived iPSCs have been shown to possess somatic donor memory,[6] in several laboratories using different methods. Existing studies show that this property would favor their differentiation propensity along donor tissue in both murine and human iPSCs.[6,48] In previous studies, this donor memory has mostly been attributed to mechanisms involving incomplete DNA demethylation as a regulator of gene expression during reprogramming.[5,10,20,48] A recent study also suggested that iPSCs possess a retaining miRNA set exclusive to their cell type of origin and that this miRNA set may contribute to somatic donor memory.[49]

To date, iPSCs have been derived from various sources including gastric epithelial cells, hepatocytes, pancreatic cells, B lymphocytes, and neural stem cells from mouse, as well as keratinocytes, skin fibroblasts, and peripheral blood
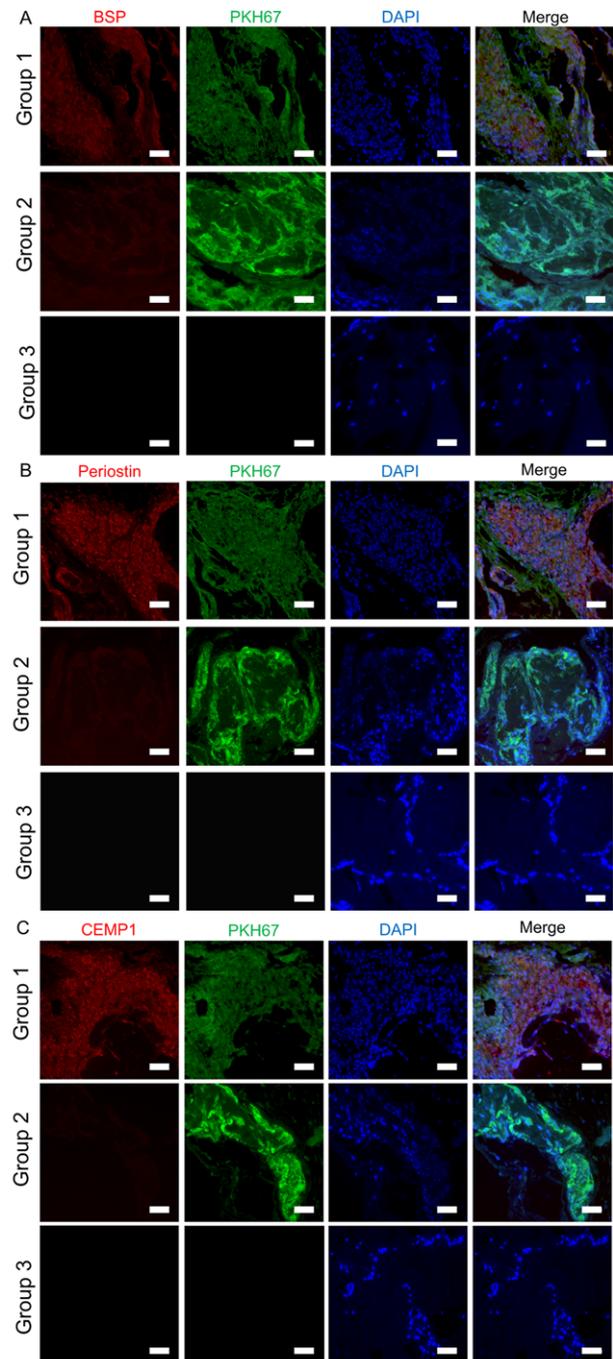

FIGURE 5 Immunofluorescence analysis of (A) BSP, (B) periostin, and (C) CEMP1 co-localization with PKH67. Most of the PKH67-labeled G-iPSCs-seeded constructs showed stronger expression compared to their S-iPSCs counterparts analyzed by Image J software (BSP: t = 8.552, $P < 0.05$; periostin: t = 3.280, $P < 0.05$; CEMP1: t = 4.902, $P < 0.05$), and their proportions of pre-labeled donor cells were 92.36% ± 8.52%, 78.21% ± 9.86%, and 86.58% ± 11.33%, respectively (n = 3 technical replicates). Marker expression was all negative in the ingrown indigenous cells. Nuclei were stained blue with DAPI. Scale bar = 100 μm. (Group 1: periodontal progenitors from G-iPSCs + hydrogel; Group 2: periodontal progenitors from S-iPSCs + hydrogel; Group 3: hydrogel only.)

cells from humans.[50] While the efficiency of inducing iPSCs into certain desired tissues has so far been shown to be enhanced by epigenetic memory, it remains a great challenge to obtain high yields of functional periodontal tissue including bone, periodontal ligaments, and cementum in a clinical setting, and whether cell type of origin has an effect on periodontal differentiation propensity remains to be determined.

In this report, we show for the first time a difference in targeted periodontal differentiation capacity between G-iPSCs and S-iPSCs based on an assay for somatic memory. Expression patterns of periodontal markers assessed at a gene and a protein level demonstrate that both iPS cell types can produce new periodontal cell populations, albeit with different efficiencies. It was shown that both G-iPSCs differentiated by induction and spontaneously express strikingly higher levels of markers relating to bone and cementum compared with S-iPSCs. The findings reported here therefore add to the growing list of somatic cell types that may exhibit donor tissue memory and test the hypothesis of the propensity of iPSCs differentiating back into periodontal tissue. The relatively minor difference in periostin expression levels between the G- and S-iPSCs may partially be attributed to additional periostin synthesis by skin fibroblasts.

To confirm our hypothesis, a rigid two-way authentication was carried out by further directing both iPSC types into skin tissue and found an overall similar tendency for spontaneous redifferentiation by the two cell types. By the same token, regular synthesis of COL-1 in gingival fibroblasts may in part account for the similar marker expression among these two iPS cell lines. Type III collagen, on the other hand, is regarded as the infantile collagen closely related to tissue elasticity and accounts for 60% of the collagen in neonatal skin, with the rest being mainly collagen I. The proportion of these two types of collagen becomes inverted during the process of growth and development.[44] Since the S-iPSCs in the present study were initially derived from newborn skin fibroblasts, their remarkably high expression level of COL-3 upon CTGF induction compared to the iPSCs derived from adult gingival tissue may indicate a residual neonatal epigenetic imprint.

Next, we investigated whether the periodontal derivatives can be directly harnessed to regenerate periodontal tissue in a subcutaneous implantation model, since stem cells have been documented to possess better survival and replication rates in vivo compared to terminally-differentiated somatic cells. A hydrogel scaffold complex is designed for the growth and differentiation of periodontal progenitors. Results showed evident staining for periodontal markers in G-iPSCs-seeded constructs, demonstrating their better maintenance of functional periodontal cell populations than S-iPSCs in vivo, which was in accordance with our in vitro findings. Differentiated cells derived from G-iPSCs may furthermore exhibit an osteoblast-like phenotype and possess a superior capacity to form mineralized deposits. When these proliferative cells were injected

into SCID mice, no teratoma tissue was developed, thus minimizing the concern over risk of teratoma formation in future clinical applications.

The capacity of iPSCs to form donor tissues was demonstrated in this study and led us to verify their retained epigenetic memory and highlight the significant implications for therapeutic use of iPSCs. It should be noted that fibroblasts mainly distribute in dense connective tissue such as the dermis and lamina propria, all derived from mesoderm; hence, comparative assays on iPSCs from other germ layers such as the ectoderm (keratinocytes) and entoderm (hepatocyte) should also be performed if possible. Accordingly, it would be informative to repeat this work using different tissue-derived iPSCs with more replicates, and to carry out more rigorous investigation via high-throughput DNA methylation arrays or whole-genome sequencing as an optimization of this study if possible. A comprehensive epigenetic characterization of these iPSC lines at different stages of reprogramming and differentiation would facilitate better elucidation of their retained lineage-specific epigenetic imprints.

In-situ regeneration animal models should also be designed to recreate a more authentic biological microenvironment for periodontal regeneration and to provide stronger evidence of functionally engrafted G-iPSC-derived periodontal cell populations, and hopefully try to obtain a satisfactory morphology of periodontal structure formation in our subsequent studies. Translating animal study findings into evidence-based therapy for patient-specific periodontal regeneration, however, should be approached with caution, and a number of measures should be taken: a facile protocol for the generation of human iPSCs from clinically available donor tissue should be established; efficient methods should be developed to drive established iPSCs into proliferative and safe periodontal cells; and scaffolds should be refined to provide beneficial microenvironments for the maintenance and promotion of functional phenotypes of the derived cells.

4 | CONCLUSIONS

To our knowledge, this is the first two-way authentication study for retained somatic memory of G-iPSCs and to the best of our knowledge, we demonstrate here for the first time that G-iPSCs exhibit a marked preference for specific periodontal differentiation. Apart from the advantage of deriving functional periodontal cell populations from G-iPSCs, gingival tissue harvesting is simpler than harvesting tissue from skin and healing of the donor site is rapid without scar formation. Although the underlying mechanisms of the donor memory of G-iPSCs needs further investigation, this additional differentiation potential adds them to the growing list of cell types that might exhibit retained donor memory, which may not only strengthen the therapeutic potential of iPSCs but avoid

treatment delay and extensive costs associated with in vitro cell culture as well. Taken together, our results indicate that human gingival fibroblasts should be considered as a superior cell source for generating patient-specific iPSCs in future regenerative periodontal therapy.

ACKNOWLEDGMENTS

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